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Construction of a biointerface on a carbon nanotube surface for efficient electron transfer

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ABSTRACT

Biodevices, which are obtained by immobilizing a biological functional molecule such as an enzyme or an antibody onto a conductive material surface, can be applied to biosensors and bio-fuel cells as a conversion and recognition element. In this study, we focus on carbon nanotubes (CNTs), which are utilized as scaffolding to facilitate electron transfer between the enzyme and electrodes. In order to achieve efficient enzymatic catalytic reactions on the CNT surface, we prepared a CNT-enzyme composite material by immobilizing an enzyme such that the active center of the enzyme was aligned with the direction of the bulk of the electrolyte solution. Multi copper oxidase from hyperthermophilic archaea *Pyrobaculum aerophilium* (Mcop) was immobilized onto the CNT surface with a high degree of orientation and the electrochemical characteristics of the resultant electrode are evaluated. The MWCNT-McoP modified electrode exhibited a current density of 190 μA/cm^2 without a mediator. Proper immobilization of McoP onto the CNT surface facilitated direct electron transfer (DET) between the CNT and McoP via the four copper atoms in McoP without a mediator.

1. Introduction[1]

Carbon nanotubes (CNTs) exhibit excellent electrical conductivity and large specific surface area, owing to their cylindrical morphology [1–3]. When they are interposed between an electrode and enzyme as a conductive wire, the electron transfer efficiency of the enzyme–electrode as well as the amount of immobilized enzyme per unit volume may be increased [4–7]. Consequently, biodevices using CNTs are expected to exhibit high output and high sensitivity [8–11].

However, owing to the hydrophobic nature of CNTs, they tend to aggregate and precipitate in aqueous solutions. Therefore, the coexistence of CNTs with biological molecules is difficult. In order to utilize the excellent inherent properties of CNTs, it is necessary to modify the surface of the CNTs such that they can be dispersed in water [12].

We previously reported a method for dispersing CNTs in water via surface modification (in submitted). In that study, we focused on pyrene derivatives, which were adsorbed onto the CNT surface through π-π stacking, thereby modifying the CNT surface. Therefore, this reaction could be exploited to obtain a CNT with a succinimide ester, which should be more hydrophilic compared with a non-treated CNT. Enzymes can be immobilized on the modified CNT surface in an aqueous system [13,14]. We have also been studying the effect of orientation of enzymes on the electrode surface on the efficiency of enzyme reactions and electron transfer at the electrode surface. By immobilizing the enzymes in an appropriate direction, the efficiency of the electrode reactions has been found to increase significantly [15].

In this study, we employed multi copper oxidase obtained from hyperthermophilic archaea *Pyrobaculum aerophilium* (Mcop) [16]. Multi copper oxidase is a generic term referring to enzymes that catalyze the four-electron oxygen reduction to produce two water molecules. McoP can be classified as type I, II, or III depending on the copper coordination and function and can oxidize a wide range of substrates. McoP has excellent heat resistance, pH stability, and exhibits long-term stability. We prepared a CNT-McoP complex as a model and evaluated its electrochemical properties. In order to

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[1] CNT: carbon nanotube; MWCNT: multi-walled carbon nanotube; Mcop: multi copper oxidase; pyrene(NHS): pyrene butyric acid N-hydroxysuccinimide ester; AB-NTA: N-(5-amino-1-carboxypentyl) iminodiacetic acid; FT-IR: fourier transform infrared spectroscopy; TEM: transmission electron microscopy; CV: cyclic voltammetry; DET: direct electron transfer

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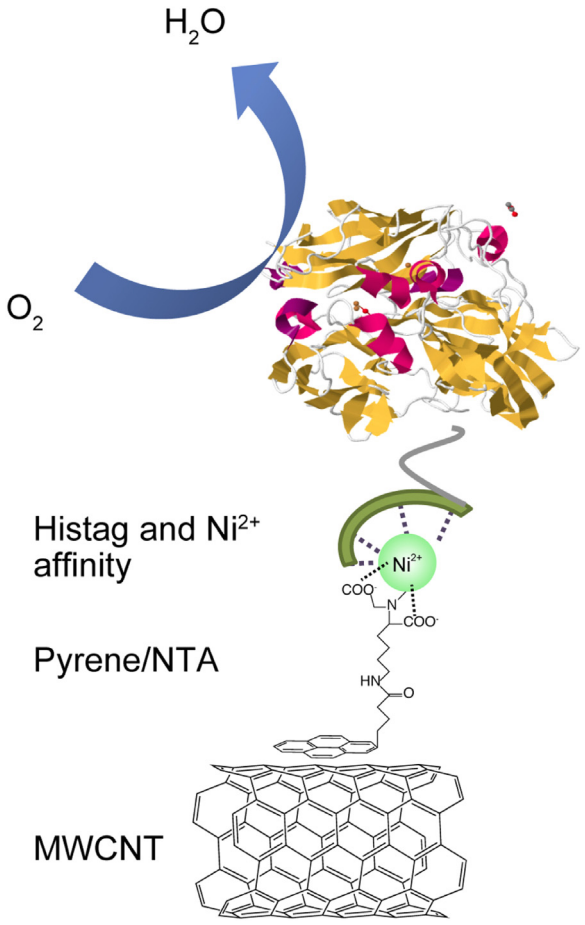

Fig. 1. Hypothetical illustration of the highly ordered immobilization of an enzyme onto a CNT surface using a pyrene derivative and His-tag affinity.

immobilize the enzyme with a high degree of orientation on the CNT surface, we utilized the affinity between His-tag and Ni^{2+} [17–19]. Therefore, His-tag (6-hisitidine residue) was genetically introduced at the N-terminal of Mcop. Subsequently, to form a Ni^{2+}-NTA complex on the CNT surface, we utilized pyrene butyric acid N-hydroxysuccinimide ester (pyrene(NHS)) and N-(5-amino-1-carboxypentyl) iminodiacetic acid (AB-NTA). The N-terminal of McoP was immobilized on the CNTs, with the active sites of McoP mainly oriented towards the bulk of the electrolyte solution. The McoP in the CNT-McoP complex was immobilized in a predetermined direction, which provided improved catalytic reaction efficiency (Fig. 1).

2. Material and methods

2.1. Reagents and instrumentation

Multi-walled carbon nanotubes (MWCNTs, $\phi = 10–30$ nm, L = 1–2 μm) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tris(hydroxymethyl) aminomethane, sodium chloride, nickel(II) sulfate hexahydrate ($NiSO_4 \cdot 6H_2O$), and sodium acetate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), whereas N-(5-amino-1-carboxypentyl)iminodiacetic acid (AB-NTA free acid) was purchased from Dojindo Laboratories (Kumamoto, Japan). 1-Pyrenebutyric acid N-hydroxysuccinimide ester [pyrene (NHS)], and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (ABTS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The water used in the experiments was first deionized and then passed through a Milli-Q water purification system from Millipore Co. (Bedford, MA, USA).

2.2. Preparation of McoP-(his-tag)

To immobilize the McoP (His-tag) with a high degree of orientation, a six-His sequence was fused at the N-terminal of the McoP. The pET15b/PAE1888 plasmid encoding McoP was used as the McoP expression vector. The expression vector of the McoP-(His-tag) fusion protein was constructed as follows. The coding region of the McoP gene was obtained by digesting pET/PAE1888 with *Nde*I/*Bam*HI [35], following which the fragment was ligated with the expression vector pET15b (Novagen, Merck Millipore Japan (Tokyo, Japan)), which was linearized with *Nde*I and *Bam*HI. The pET15/PAE1888 plasmid was verified by DNA sequencing.

McoP-(His-tag) was expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL (Toyobo, Tokyo, Japan) grown in an LB medium containing ampicillin. Recombinant McoP-(His-tag) proteins were induced by adding 1 mM isopropyl-β-D-thiogalacto-pyranoside at OD_{600} 0.6, and cultivation was continued for an additional 21 h at 20ºC. The cells were harvested by centrifugation at 8000 × *g*, suspended in lysis buffer, and disrupted by ultrasonication. The cell debris was removed by centrifugation (15,000 × *g* for 10 min), and the supernatant solution was used as the crude extract. The enzyme solution was then incubated at 80 °C for 10 min and the denatured proteins were separated by centrifugation (15,000 × *g* for 10 min). The resultant supernatant was subjected to further purification.

McoP-(His-tag) was purified using Ni-NTA chromatography (HisTrap HP column; GE Healthcare UK Ltd, Little Chalfont, Bucks, UK). The enzyme was loaded onto the column equilibrated with 50 mM HEPES buffer at pH 7.5 and eluted from the column using an elution buffer (100 mM acetate buffer at pH 3.0).

The activity of McoP-(His-tag) was measured using ABTS, as described previously [16]. One unit was defined as the amount of the McoP that oxidized 1μmol of ABTS substrate per min.

2.3. Dispersion of CNTs

To purify the CNTs, they were placed in 4 M HCl for 12 h. The CNTs were collected by suction filtration and washed with 50 mM HEPES-NaOH buffer (pH 7.5). Approximately 1 mg of CNTs and 3 mM pyrene (NHS) were sonicated for 3 h in a bath-type sonicator in HEPES buffer to obtain CNT/pyrene(NHS).

2.4. Enzyme immobilization onto the dispersed CNTs

In order to impart the CNTs with Ni^{2+} holding capacity, we decorated the CNT surface with an NTA linker. AB-NTA (10 mM) was added into a 1 mL CNT dispersion solution, which was incubated for 2 h at room temperature (CNT/pyrene(NHS)AB-NTA). Subsequently, 50 mM $NiCl_2$ was added to the mixture, which was incubated for an additional 30 min. The unreacted substrate was removed by centrifugation (12,000 × *g* for 10 min). The CNT pellets were washed twice in 50 mM HEPES-NaOH buffer (pH 7.0), resuspended in 1 mL of the enzyme solution (2.5 mg/mL), and incubated for 5 min (CNT/pyrene(NHS)/Ni-NTA/McoP). The washing step was repeated and the complex obtained was stored at room temperature until further use. The activity of the CNT–enzyme complex was measured ABTS, as described previously [16].

2.5. Fourier transform infrared spectroscopy (FT-IR)

FT-IR measurements were conducted using a Thermo Nicolet 6700 FT-IR spectrometer (Madison, WI, USA) equipped with a